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Piper aduncum against *Haemonchus contortus* isolates: cross resistance and the research of natural bioactive compounds

Piper aduncum contra isolados de *Haemonchus contortus*: resistência cruzada e a pesquisa de bioativos naturais

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Abstract

The anthelminthic activity of the essential oil (EO) of *Piper aduncum* L. was tested *in vitro* on eggs and larvae of resistant (Embrapa2010) and susceptible (McMaster) isolates of *Haemonchus contortus*. The EO was obtained by steam distillation and its components identified by chromatography. EO concentrations of 12.5 to 0.02 mg/mL were used in the egg hatch test (EHT) and concentrations of 3.12 to 0.01 mg/mL in the larval development test (LDT). Inhibition concentrations (IC) were determined by the SAS Probit procedure, and significant differences assessed by ANOVA followed by Tukey's test. In the EHT, the IC_{50} for the susceptible isolate was 5.72 mg/mL. In the LDT, the IC_{50} and IC_{90} were, respectively, 0.10 mg/mL and 0.34 mg/mL for the susceptible isolate, and 0.22 mg/mL and 0.51 mg/mL for the resistant isolate. The EO (dillapiole 76.2%) was highly efficacious on phase L_1. Due to the higher ICs obtained for the resistant isolate, it was raised the hypothesis that dillapiole may have a mechanism of action that resembles those of other anthelmintic compounds. We further review and discuss studies, especially those conducted in Brazil, that quantified the major constituents of *P. aduncum*-derived EO.

Keywords: Isolates, *Haemonchus contortus*, cross resistance, dillapiole, synergy.

Resumo

Este estudo avaliou a atividade anti-helmíntica *in vitro* do óleo essencial (OE) de *Piper aduncum* L. sobre ovos e larvas de *Haemonchus contortus*, verificando se um isolado resistente (Embrapa2010), apresentaria o mesmo comportamento que um sensível (McMaster). O OE foi obtido por arraste a vapor e analisado por cromatografia para identificação dos constituintes. O óleo foi avaliado nas concentrações de 12,5 a 0,02 mg/mL no Teste de eclosão dos ovos (TEO) e nas concentrações de 3,12 a 0,01 mg/mL no Teste de desenvolvimento larvar (TDL). As concentrações inibitórias (CI) foram determinadas pelo procedimento Probit do SAS e as diferenças estatísticas geradas pela ANOVA seguida pelo teste de Tukey. Para o isolado sensível obteve-se CI_{50} de 5,72 mg/mL no TEO. No TDL o óleo apresentou CI_{50} e CI_{90} de 0,10 mg/mL e 0,34 mg/mL para o isolado sensível e 0,22 mg/mL e 0,51 mg/mL para o resistente, respectivamente. Demonstrou-se que o OE (dilapiol 76,2%) teve alta eficácia sobre a fase L_1. Devido às elevadas CIs obtidas para o isolado resistente, levantou-se a hipótese de que o dilapiol talvez possua um mecanismo de ação semelhante a algum grupo anti-helmíntico. O artigo faz uma revisão e discute estudos de quantificação dos constituintes majoritários do OE de *P. aduncum*, destacando os realizados no Brasil.

Palavras-chave: Isolados, *Haemonchus contortus*, resistência cruzada, dilapiol, sinergismo.

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Introduction

The nematode *Haemonchus contortus* parasitizes the gastrointestinal tract of ruminants, causing anemia, weight loss, and, in severe infections, death (URQUHART et al., 1998). *Haemonchus* infections affect hundreds of millions of small ruminants worldwide causing estimated losses of US$ 10 billion per year (WALLER & CHANDRAWATHANI, 2005; ROEBER et al., 2013). The use of synthetic anthelmintic substances constitutes the main course of action against gastrointestinal parasitism (FREEMAN et al., 2003). However, resistance to currently available anthelmintic drugs drastically reduces the effects of parasite control programs (JAMES et al., 2009; SUTHERLAND & LEATHWICK, 2011). Therefore, phytotherapeutic strategies have become the target of researchers aiming at developing environmentally-friendly tools for the control of nematodes in small ruminants.

Secondary metabolites probably mediate many of the biological activities of medicinal plants. These metabolites have become an alternative treatment, especially in organic farming where regulation forbids the routine use of synthetic anthelmintic drugs (CHAGAS, 2004; LISONBEE et al., 2009). Among such compounds, essential oils (EOs) represent an important category. They can be obtained from hydro- or dry distillation of plant material (ISO, 2013), and consist of a mixture of volatile, lipophilic, often odoriferous and liquid substances. Interaction among these substances may interfere in nematode metabolism, inhibiting and disorganizing vital functions since the early stages of development (OKA et al., 2000).

Some plant species, when processed, yield large amounts of EOs, especially those in the Apiaceae, Asteraceae, Cupressaceae, Hypericaceae, Clusiaceae, Lamiaceae, Lauraceae, Fabaceae, Liliaceae, Myrtaceae, Pinaceae, Piperaceae, Rosaceae, Rutaceae, Santalaceae, Zingiberaceae and Zygophyllaceae families (HUSNU et al., 2007; FIGUEIREDO et al., 2008). Specifically, Piperaceae family EOs have been tested in many different contexts worldwide (GUPTA, 1995; OVIEDO-RONDON et al., 2006; SRINIVASAN, 2007; SCOTT et al., 2008; GUERRINI et al., 2009; SILVA et al., 2009; SINGH et al., 2009; PINO et al., 2011; ARAUJO et al., 2012). The biological activity of the *Piper* genus, native to tropical and subtropical regions (JARAMILLO & MANOS, 2001), has been more extensively studied. Within this genus, the spiked pepper *Piper aduncum* L. produces a biodegradable EO with high commercial value due to its fungicidal, bactericidal, molluscicidal, acaricidal, insecticidal and larvicidal activities (MAIA et al., 1998). The species has a high tolerance to different weather conditions and can be cultured in a wide range of areas (LORENZI & MATOS, 2002; SOUSA et al., 2008). Finally, spiked pepper cultures may represent a crucial stepping stone for regional economic development based on a renewable source of chemical raw materials, which can be processed in large or small scales (FAZOLIN et al., 2006).

The distinct biological activities attributed to the varying chemical composition of *P. aduncum* EO fuel studies on the potential veterinary applications of this species. Thus, the present work aims at assessing the *in vitro* anthelmintic activity of *P. aduncum* EO on eggs and larvae of resistant and susceptible isolates of *H. contortus*. Because these parasites had never been exposed to the plant extract, we assessed if the resistant isolate would present similar response that of susceptible isolate.

Materials and Methods

P. aduncum cultivation and EO extraction

P. aduncum plants were cultivated at Embrapa Amazônia Ocidental, located in the city of Manaus, state of Amazonas, Brazil, in 2014. The cultivation took place in field plots with soil classified as Dystrophic Yellow Latosol, which was fertilized with chicken manure (1.0 kg m^2) added before cultivation. The field plots were located at the geographic coordinates 03º 06' 23.04" S and 60º 01' 35.14" W, at an average altitude of 50 m, average temperature of 25.6 °C, and average annual rainfall of 2,200 mm. The equatorial climate is characterized as Af, according to the world map of the Köppen-Geiger climate classification. Samples of *P. aduncum* were deposited in the EAFM herbarium of the Amazon Federal Institute under the number 10.480.

After harvest, in July 2014, leaves were separated from branches and shade-dried, in a barn. After drying for one week, leaves were taken to the laboratory where extraction was conducted by hydro-distillation in a modified Clevenger-type apparatus.

EO chromatographic analyses

Chemical composition analysis of the EO was conducted at Embrapa Agroindústria de Alimentos, Rio de Janeiro, Brazil, with gas chromatography coupled to mass spectrometry (GC-MS) in an Agilent 5973 N system (Agilent Technologies, DE, USA) equipped with a HP-5MS capillary column (5%-diphenyl, 95%-dimethyl-silicone, 30 m × 0.25 mm; film thickness 0.25 µm). Helium was used as the carrier gas (1.0 mL min^{-1}). A total volume of 1.0 µL of a 1% solution of the oil in dichloromethane was injected into an injector heated to 250 °C, operating in split mode (split ratio 1:100). Oven temperature was raised from 60 °C to 240 °C, at a rate of 3 °C min^{-1}. The mass detector was operated in electronic ionization mode (70 eV) with the mass analyzer maintained at 150 °C, the ionization source at 220 °C, and the transfer line at 260 °C. Linear retention indices were calculated by injection of a series of n-alkanes (C_7-C_{26}) in the same column and conditions as above. Identification of the essential oil components was done by comparison of both mass spectra and retention indices with the Wiley sixth edition spectral database and other data from the literature (ADAMS, 2007).

For quantification, the essential oil was analyzed in an Agilent 7890A chromatograph equipped with a flame ionization detector (FID) kept at 280 °C and fitted with an HP-5MS capillary column (5% diphenyl-95%-dimethyl-silicone; 30 m× 0.25 mm; film thickness 0.25 µm). The same injection and chromatographic conditions described above were applied, but hydrogen was used as the carrier gas at 1.5 mL min^{-1}. Results were expressed as relative area (percentage area).

Donor animals and isolates of H. contortus

Four Santa Inês animals were obtained from the ovine herd of Embrapa Pecuária Sudeste (CPPSE), São Carlos/SP. These animals had between three and four months of age, and weighed an average of 30 kg. They were initially treated with Zolvix® (monepantel, 2.5 mg/kg body weight) for the removal of natural parasitic infections. Seven and 14 days after deworming, we performed fecal counts of eggs per gram (EPG) to be sure the animals were clean of nematodes. Fecal material was collected directly from the rectum and eggs were enumerated using the McMaster x 50 technique (ROBERTS & O'SULLIVAN, 1950). Then, two animals were mono-specifically infected with 4000 L_3 of the isolate *H. contortus* McMaster and the other two with 4000 L_3 the isolate *H. contortus* Embrapa2010. Each pair of animals was placed in separate stalls, supplemented *ad libitum* with corn silage, water and mineral salt. All procedures involving donor animals were approved by the Committee on Animal Research and Ethics of the CPPSE (Protocol no. 06/2012).

H. contortus McMaster displays susceptibility to numerous chemical groups and has no history of exposure to anthelminthic agents (GILL et al., 1995). *H. contortus* Embrapa2010 was originally isolated from the CPPSE herd, and cryopreserved since 2006. The isolate was registered in the anthelmintic resistance consortium SNPs-parasite isolate database (CARS). It has displayed resistance to benzimidazole (20% efficacy), ivermectin (52%), moxidectin (85%), levamisole (93%) and high susceptibility to closantel (100%) and monepantel (100%) (CHAGAS et al., 2013).

In vitro tests

The *P. aduncum* EO was assessed in the egg hatch test (EHT) in 12 concentrations (0.02, 0.04, 0.09, 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 mg/mL) and in the larval development test (LDT) in seven concentrations (0.04, 0.09, 0.19, 0.39, 0.78, 1.56 and 3.12 mg/mL). To improve EO emulsification in water, Tween 80 at 2% was added in the EHT and DMSO at 0.5% in the LDT and the solutions were homogenized in a vortex mixer until oil, solvent and water formed a stable emulsion.

Recovery of H. contortus eggs

Fresh feces from the donor animals described above were obtained for the isolation of nematode eggs, according to the method described by Coles et al. (1992), and adapted by Chagas et al. (2011). In summary, approximately 5 g of feces, directly collected from the rectum, were mixed with tepid water (37 °C) and filtered through sieves with mesh sizes of 1 mm, 106 µm, 53 µm and 25 µm. The last and finer mesh retained the eggs. Recovered eggs were added to a saturated NaCl solution, centrifuged at 3000 rpm for 5 min, then floating eggs were collected using a 25 µm sieve and washed with distilled water. Eggs were separated and quantified.

EHT

One hundred eggs in 10 µL of distilled water were added to each well of a 24-well microplate. Then the EO, water (negative control 1), 2% Tween 80 (negative control 2) or thiabendazole (Sigma, T8904) at 60 µg/mL (positive control) was added to the wells. Each EO concentration and each control had six repetitions performed in three independent experiments, making 18 wells for each treatment or approximately 1800 eggs evaluated. Each line/treatment of 24-well plates was identified. Then the plate was sealed with PVC film and incubated at 27 °C and ≥ 80% relative humidity for 24 h. After this time, eggs and L_1 larvae were counted with an inverted microscope to calculate the egg hatching inhibition (CHAGAS et al., 2011).

LDT

As described in Chagas et al. (2011), 100 eggs were added to each well of a 24-well microplate with distilled water for a total volume of 250 µL in each well. The plates were incubated for 24 h at 27 °C and ≥80% relative humidity to obtain L_1 larvae. After this time, nutritive medium was added to each well according to Hubert & Kerboeuf (1992). All concentrations of essential oils, water (negative control 1), DMSO at 0.5% (negative control 2), and ivermectin (Sigma, I8898) at 10 µg/mL (positive control) were tested in six repetitions performed in three independent experiments (approximately 1,800 L_1 evaluated/treatment). The plates were incubated under the same conditions for six days, when each well was analyzed with an inverted microscope and all L_3 and undeveloped larvae were counted, to estimate larval development inhibition.

Statistical analysis

In the EHT and LDT, the percentage of inhibition or efficacy of each treatment was determined based on the arithmetic mean (X) of the egg hatching or larval development, according to the following equation (COLES et al., 1992):

$$\text{Inhibition } (\%) = 100 \left(X_{Test} / X_{total} \right) \quad (1)$$

where, X_{Test} represents the number of unhatched eggs in the EHT, or the number of larvae that did not develop until L_3 in the LDT, and X_{total} corresponds to the total number of eggs + L_1 in the EHT, or the number of L_1+ L_2+ L_3 in the LDT.

The efficacy data were subjected to a variance analysis by the GLM procedure of SAS following a randomized experimental design with two isolates (resistant and susceptible) and 13 levels of concentration, describing a factorial 2 x 13, with 6 repetitions. We studied the effect of isolate, concentration and their interaction "ISOxCONC". Then it was performed a Tukey's multiple comparison test (SAS 9.0 for Windows) in order to detect significant statistical differences ($p \leq 0.01$). The calculation of the inhibition concentrations (ICs) was performed by the SAS Probit procedure to estimate the IC_{50} and IC_{90}.

Results

We obtained an average yield of *P. aduncum* EO of 3.6% (v/w). The EO chemical composition and retention times are indicated in Table 1. We identified a total of 36 substances, representing

99.1% of the oil extracted from the leaves. Dillapiole represented the major compound (76.2%), followed by (*E*)-caryophyllene (3.8%) and myristicin (3.6%).

Results from the EHT indicated that the EO had better effect on the susceptible isolate. At the two lowest concentrations, the EO was not efficacious and results were statically similar to negative controls (2% tween 80 and distilled water). On the other hand, better effects were seen in the highest concentrations; at 0.39, 0.78, 1.56 and, 3.12 mg/mL results of egg inhibition were statistically similar. In the case of the resistant isolate Embrapa2010, the highest EO effect was seen in the concentration 12.5 mg/mL, which was statistically similar to the concentrations 6.25 and 3.12 mg/mL. Again, the EO results were statically similar to negative controls at the two lowest concentrations. When the isolates were compared in the same concentration, all results were significantly different ($p \leq 0.01$) (Table 2). The highest EO concentration (12.5 mg/mL) inhibited hatching by 55.80% of the susceptible eggs, and by 14.68% of the resistant eggs. The dose-response was linear for susceptible eggs, and the IC_{50} was calculated at 5.72 mg/mL (Table 3). Because of the relatively low effect of the EO on egg hatching, we could not define the ICs for the resistant isolate, or the IC_{90} for the susceptible one.

The percentage inhibitions in larval development from L_1 to L_3 mediated by the EO are displayed on Table 4. At the three highest concentrations, the EO was highly efficacious, inhibiting larval development of the two *H. contortus* isolates by more than 99%. There was no significant difference among concentrations from 0.37 to 3.12 mg/mL in the McMaster isolate. The same occurred among concentrations from 0.78 to 3.12 mg/ml to the Embrapa2010 isolate. LDT results were also significantly different

Table 1. Composition of the essential oil of *Piper aduncum* L. assessed by GC/MS, retention time (t_R subst) and linear retention index available in the literature (LRI).

Compound	LRI	% relative
α-pinene	931	0.5
β-pinene	974	1.7
myrcene	989	0.1
α-phellandrene	1003	0.2
limonene	1025	0.6
(*Z*)-β-ocimene	1034	1.0
(*E*)-β-ocimene	1045	2.2
γ-terpinene	1054	0.1
linalool	1100	0.2
α-terpineol	1189	0.1
n.i.	1210	0.1
ciclosativene	1359	0.1
α-copaene	1368	0.2
β-elemene	1385	0.2
α-gurjunene	1401	0.1
(*E*)-caryophyllene	1412	3.8
β-copaene	1420	0.1
α-*trans*-bergamotene	1429	0.1
α-humulene	1445	0.6
allo-aromadendrene	1452	0.2
germacrene D	1472	0.9
ar-curcumene	1477	0.1
trans-4(14),5-muuroladiene	1482	0.1
epi-cubebol	1487	0.6
α-muurolene	1493	0.4
cubebol	1508	0.4
myristicin	1521	3.6
n.i.	1535	0.1
germacrene B	1546	0.2
isoelemencine	1557	0.3
(*E*)-nerolidol	1561	0.9
spathulenol	1571	1.7
viridiflorol	1584	1.0
ledol	1593	0.2
humulene epoxide II	1599	0.1
n.i.	1602	0.2
n.i.	1614	0.1
dillapiole	1640	76.2
α-cadinol	1654	0.4
n.i.	1667	0.3
apiole	1680	0.3
Total identified		**99.1**

n.i.: not identified.

Table 2. Inhibition efficacy (mean percentage ± S.E.) of the essential oil extracted from *Piper aduncum* L. (mg/mL) on hatching of eggs from susceptible McMaster and resistant Embrapa2010 isolates of *Haemonchus contortus* in the egg hatch test (EHT). Also shown are controls containing water, Tween 80 and thiabendazole.

Concentrations	McMaster	Embrapa2010
0.02	2.68 ± 0.31 ^{Ab}	1.10 ± 0.15 ^{Aa}
0.04	7.03 ± 0.35 ^{ABb}	2.53 ± 0.21 ^{ABa}
0.09	12.10 ± 0.44 ^{Bb}	4.63 ± 0.19 ^{BCa}
0.19	23.04 ± 0.85 ^{Cb}	6.58 ± 0.41 ^{CDa}
0.39	35.13 ± 1.03 ^{Db}	8.34 ± 0.62 ^{DEa}
0.78	37.04 ± 0.80 ^{Db}	9.57 ± 0.29 ^{EFa}
1.56	37.55 ± 1.05 ^{Db}	11.32 ± 0.54 ^{FGa}
3.12	38.73 ± 0.66 ^{DEb}	12.85 ± 0.72 ^{GHa}
6.25	44.80 ± 2.25 ^{Eb}	13.88 ± 0.65 ^{GHa}
12.5	55.80 ± 2.63 ^{Fb}	14.68 ± 0.67 ^{Ha}
2% Tween 80	2.35 ± 1.08 ^{ABa}	2.47 ± 0.19 ^{ABa}
Distilled water	1.92 ± 1.08 ^{ABa}	1.67 ± 0.23 ^{Aa}
Thiabendazole	100 ± 0.00 ^{Ga}	100 ± 0.00 ^{Ia}

Different upper-case letters in the column (concentrations) and different lower-case letters in the line (isolates) indicate significant differences according to Tukey's test ($p \leq 0.01$).

Table 3. IC_{50}, IC_{90} (mg/mL) and confidence intervals obtained in the egg hatch test (EHT) and larval development test (LDT) using essential oil extracted from *Piper aduncum* L. against susceptible McMaster and resistant Embrapa2010 isolates of *Haemonchus contortus*.

Test	Isolates	IC_{50}	IC_{90}
EHT	McMaster	5.72 (4.81 - 6.93)	*
	Embrapa2010	*	*
LDT	McMaster	0.10 (0.09-0.11)	0.34 (0.32-0.37)
	Embrapa2010	0.22 (0.21-0.23)	0.51 (0.48-0.54)

*Could not be estimated.

Table 4. Inhibition efficacy (mean percentage ± S.E.) of the essential oil extracted from *Piper aduncum* L. (mg/mL) on larval development of susceptible McMaster and resistant Embrapa2010 isolates of *Haemonchus contortus* in the larval development test (LDT). Also shown are controls containing water, DMSO and ivermectin.

Concentrations	McMaster	Embrapa2010
0.01	3.12 ± 0.33 ^{Ab}	0.47 ± 0.32 ^{Aa}
0.02	12.16 ± 0.32 ^{Bb}	1.91 ± 0.35 ^{Aa}
0.04	20.59 ± 0.69 ^{Cb}	6.00 ± 0.45 ^{Ba}
0.09	41.41 ± 0.52 ^{Db}	13.11 ± 0.46 ^{Ca}
0.19	60.26 ± 0.97 ^{Eb}	24.37 ± 0.78 ^{Da}
0.37	99.37 ± 0.40 ^{Fb}	85.58 ± 1.09 ^{Ea}
0.78	100 ± 0.00 ^{Fa}	99.52 ± 0.32 ^{Fa}
1.56	100 ± 0.00 ^{Fa}	99.68 ± 0.20 ^{Fa}
3.12	100 ± 0.00 ^{Fa}	100 ± 0.00 ^{Fa}
0.5% DMSO	2.33 ± 0.05 ^{Aa}	2.90 ± 0.27 ^{ABa}
Distilled water	1.86 ± 0.02 ^{Aa}	2.28 ± 0.21 ^{ABa}
Ivermectin	100 ± 0.00 ^{Ga}	100 ± 0.00 ^{Ga}

Different upper-case letters in the column (concentrations) and different lower-case letters in the line (isolates) indicate significant differences according to Tukey's test ($p \leq 0.01$).

between the two isolates ($p \leq 0.01$) at every concentration except for the three highest ones. A dose-response relation was observed for both isolates (Table 4).

The ICs for both isolates calculated from the LDT are summarized in Table 3. The IC_{50} for the susceptible isolate was 0.10 mg/mL, and it reached an approximately twofold higher value (0.22 mg/mL) for the resistant isolate.

Discussion

An estimated 500 thousand plant species exist in the world, and 16% of them are in the Amazon forest in Brazil. The diversity of this country's flora represents an enormous reservoir of mostly unexplored plant secondary compounds. Despite increasing research efforts to unveil new species and understand their roles over the past two decades, our knowledge remains scant (FAZOLIN et al., 2007). Nevertheless, different industrial sectors have increasingly sought Amazon plant compounds, including the agro-business sector.

Few reports discuss the potential of EOs derived from *P. aduncum* L. against nematodes that parasitize ruminants. In the present study, EO anti-hatching activity was only evident against susceptible eggs at the highest concentration (55.8% inhibition with 12.5 mg/mL), which was statistically different of the other concentrations on McMaster isolate, as well as at the same concentration (12.5 mg/mL) on the Embrapa2010 isolate. Other work using the EHT showed that the EO from *P. aduncum* L. was 95% efficacious at 12 mg/mL against *H. contortus* eggs (OLIVEIRA et al., 2014). We believe this divergent results stem from the chemical composition of EOs, which in the previous study primarily contained 1,8-cineole (55.8% against 0% dillapiole), whereas in the present study 76.2% of the oil was composed of dillapiole with no 1,8-cineole. Qualitative and quantitative differences in plant composition result from several factors including genetic background and plant variety (BARBOSA et al., 2007; HABER, 2008), site of cultivation (HABER, 2008; FURLAN et al., 2010), climatic and agricultural conditions (MARCO et al., 2007), harvest season and timing (BLANK et al., 2007; FURLAN et al., 2010), as well as plant developmental stage (LAHLOU & BERRADA, 2003; LEAL et al., 2003). The extraction method (e.g., super-citric fluid, steam distillation, solvent) also affects oil composition (QUISPE-CONDORI et al., 2008), much like drying (BLANK et al., 2007; BARBOSA et al., 2008) and storage conditions (GUIMARÃES et al., 2008).

In contrast to the mild effects on egg hatching, the EO significantly inhibited larval development of both isolates in a dose-dependent manner. Eggs in their hard and resistant shell usually resist chemicals to a larger extent than L_1 larvae (KATIKI et al., 2011). At the four highest concentrations the EO was more than 99% efficacious in inhibiting larval development of the susceptible isolate and, at three highest concentrations of the resistant isolate. For both isolates the results were statistically similar at those concentrations. When isolates were compared at the same concentration, the larval development inhibition was statistically similar at 0.78, 1.56 and 3.12 mg/mL. The IC_{50} represented low EO doses for both isolates, but the dose required to kill 50% of the resistant larvae was twofold greater than required for the susceptible ones. This *in vitro* effect observed with the LDT may be related to the arylpropanoid dillapiole, to which many of the pharmacological activities of the EO extracted from *P. aduncum* L. have been attributed (MAIA et al., 1998; POHLIT et al., 2006). In fact, dillapiole has been shown to have acaricidal (SILVA et al., 2009; PINO et al., 2011; ARAÚJO et al., 2012), anti-bacterial (LARA-JÚNIOR et al., 2012; BRAZÃO et al., 2014), anti-fungal (LARA-JÚNIOR et al., 2012; GUERRINI et al., 2009), leishmanicidal (PARISE-FILHO et al., 2012), anti-inflammatory (PARISE-FILHO et al., 2011) and insecticidal activities (BELZILE et al., 2000; SOUTO, 2006; LING et al., 2009). When 99% pure, this compound has been evaluated and identified as the mediator of many of the biological activities above (ALMEIDA, 2004).

Dillapiole may also associate with other minor bioactive compounds in the EO potentiating their action. For example, myristicin, present at 3.6% in the tested EO, sarisan (BIZZO et al., 2001) or safrole (HUANG et al., 1999), all of which have a methylenedioxyphenyl group in their structure are natural insecticides that could be the target of such association. Brazão et al. (2014), when assessing the EO extracted from *P. aduncum* against susceptible and resistant isolates of *Staphylococcu*s spp., also showed the combined bactericidal action of dillapiole and myristicin (1 to 2%), lending further support to our conclusions.

Previous work revealed dillapiole as the major component of the EO of different varieties of *P. aduncum* collected in the Amazon, such as *P. aduncum* L. (MAIA et al., 1998; ALMEIDA et al., 2009), *P. aduncum var. aduncum* and *P. aduncum var. cordulatum* (GOTTLIEB et al., 1981). However, individuals from this same species collected in other regions have other major constituents, including 1,8-cineole (LARA-JÚNIOR et al., 2012; OLIVEIRA et al., 2013), (*E*)-nerolidol (MESQUITA et al., 2005; OLIVEIRA et al., 2006) and nerolidol (NAVICKIENE et al., 2006), asaricine (FERREIRA, 2011; POTZERNHEIM et al., 2012) and piperitone

Table 5. Major chemical compounds making up the essential oils from *Piper aduncum* L. leaves, as reported by different authors.

Chemical compound	Relative abundance (%)	Origin	Reference
β-caryophyllene	17.4	Panama City/Panama	Vila et al. (2005)
Dillapiole	86.9	Amazonas/Brazil	Almeida et al. (2009)
	45.9	Morona/Ecuador	Guerrini et al. (2009)
	43.3	Morobe/Papua New Guine	Rali et al. (2007)
	82.2	Pinar do Río/Cuba	Pino et al. (2004)
	31.5	Amapa/Brazil	Maia et al. (1998)
	50.8-86.9	Para/Brazil	
	91.1-97.3	Amazonas/Brazil	
	88.1	Acre/Brazil	
	64.5	Kuala Lumpur/Malaysia	Jantan et al. (1994)
	90.0	Panama	Gupta et al. (1983)
	58.0	Fiji	Smith & Kassim (1979)
	61.8	Cartago y San José/ Costa rica	Cicció & Ballestero (1997)
	74.5-88.4	Amazonas/Brazil	Gottlieb et al. (1981)
	49.5	Brasilia/Brazil	Potzernheim et al. (2012)
1,8-cineole	40.5	Cochabamba/Bolivia	Vila et al. (2005)
	42.0-42.5	Cochabamba/Bolivia	Arze et al. (2008)
	48.0-53.9	Minas Gerais /Brazil	Lara-Júnior et al. (2012)
	23.3-57.2	Minas Gerais/Brazil	Oliveira et al. (2013)
(*E*)-nerolidol	79.2-82.5	Pernambuco/Brazil	Oliveira et al. (2006)
	14.2	Minas Gerais/Brazil	Mesquita et al. (2005)
Nerolidol	10.4	São Paulo/Brazil	Navickiene et al. (2006)
Asaricin	10.5	Cochabamba/Bolivia	Arze et al. (2008)
	49.0	São Paulo/Brazil	Ferreira (2011)
	15.8	Brasilia/Brazil	Potzernheim et al. (2012)
Piperitone	22.7-24.9	Brasilia/Brazil	Potzernheim et al. (2012)

(POTZERNHEIM et al., 2012). Table 5 summarizes the major components found in leaf EOs from *P. aduncum* L., highlighting the results of studies conducted in Brazil. The Piperaceae taxonomy is very complex (KATO & FURLAN, 2007). For different reasons, *P. aduncum* L. was botanically described in the Neotropical region by several authors in distinct times and places. In Brazil, there also were diverging points of view as to its taxonomic identity, and two varieties or subspecies were described by Gottlieb et al. (1981): *P. aduncum var. aduncum* and *P. aduncum var. cordulatum.* Recently, a single nomenclature – *P. aduncum var. aduncum* – was adopted (GUIMARÃES et al., 2015).

The chemical variation observed in the EO of *P. aduncum* L. may result from the predominance of different biosynthetic pathways in two chemotypes (ALMEIDA et al., 2009). In the Amazon forest the shikimate pathway took precedence, producing the phenylpropanoid dillapiole, whereas in the Atlantic forest chemotype, the mevalonate/acetate pathway was favored, generating the terpenoids (*E*)-nerolidol and linalool. These authors showed the occurrence of both chemotypes in both ecosystems, with their individual predominance depending on environmental factors. In addition, Andrade et al. (2009) conducted a hierarchical analysis based on EO chemical composition and clustered 21 *P. aduncum* specimens into four groups, from A to D. The EO examined in the present study fits the A chemical profile of the Amazon *P. aduncum* variety. This complex taxonomy and classification system indicates that differences in results may well stem from misclassifications at the subspecies level. Moreover, in face of such complexity, the phytochemical study of each oil's efficacy against parasites becomes of paramount importance. Every pharmaceutic and veterinary product must be chemically qualified, i.e., the EOs must have known compositions. Bioactive markers must be established and, for any given use, the EOs must have these markers within the appropriate range to elicit the expected effects. Thus, we propose that the stages of taxonomic identification, extraction and phytochemistry should be clearly developed for *P. aduncum* to further define its anthelminthic activity, as shown in Figure 1.

The action of *P. aduncum* extracts on *H. contortus* may be explained at the molecular level as an effect of the methylenedioxyphenyl group present in dillapiole (Figure 2) and in other substances of the Piperaceae-derived EOs (FAZOLIN et al., 2007). This group

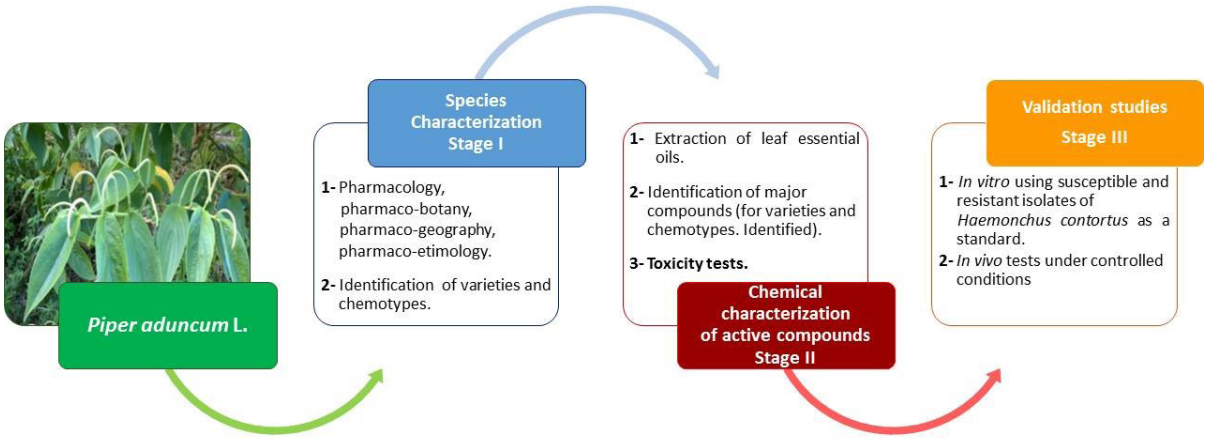
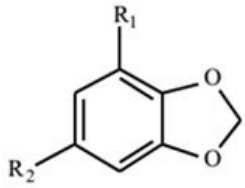


Figure 1. Required steps in the research of essential oils derived from *Piper aduncum* L. as anthelmintic agent against *Haemonchus contortus*.



R_1	R_2	
CH_3O-	CH_3O-	Dillapiole
CH_3O-	H_2C=CH-CH_2-	Myristicin
CH_2-O-CH_2-CH_2-O-CH_2-CH_2-O-C_4H_9-	CH2-CH2-CH3-	Piperonyl butoxide

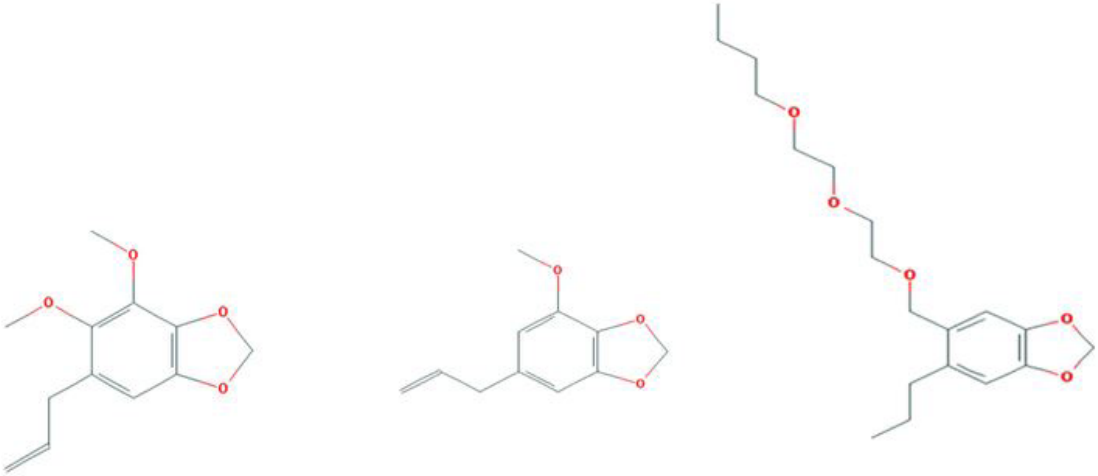


Figure 2. Major aryl-propanoids present in the EO of *Piper aduncum*, highlighting the presence of a methylenedioxyphenyl group and its structural analogy to piperonyl butoxide.

represents an important inhibitor of cytochrome P450-dependent monooxygenases (MUKERJEE et al., 1979; BERNARD et al., 1990; FAZOLIN et al., 2014). Monooxygenase inhibition reduces the detoxification capacity of insects, which are then poisoned by plant compounds that would otherwise be gradually eliminated (BERNARD et al., 1995). According to Kotze et al. (2006), piperonyl butoxide, which is related to dillapiole, acts synergistically with insecticides by inhibiting cytochrome P450-mediated metabolism of the insecticide. In the same study, the anti-helminthic properties of rotenone and its activity in combination with piperonyl butoxide, were examined *in vitro*. Rotenone was toxic to larvae of *H. contortus* and *Trichostrongylus colubriformis*, with IC_{50} values in the LDT of 0.54 and 0.64 µg/mL, respectively. The compound also caused complete cessation of movement in adult *H. contortus* at 20 µg/mL. Rotenone toxicity towards larvae and adults increased in the presence of piperonyl butoxide. Thus, the significant synergism observed suggests that these two nematode species use a cytochrome P450 pathway to detoxify rotenone, and indicates that a role may exist for cytochrome P450 inhibitors, such as piperonyl butoxide, to potentiate the action of anthelminthic substances that undergo oxidative metabolism within the nematode (KOTZE et al., 2006). The role of oxidative pathways in the metabolism of benzimidazoles in susceptible and resistant nematodes is unknown. Thus, the ability of a synergist to increase the toxicity of this chemical group towards both susceptible and resistant strains remains to be determined (KOTZE et al., 2006). Resistance to macrocyclic lactones does not involve enhanced oxidative metabolism and will likely require other types of assessments to have its mechanism determined (KOTZE, 2000).

In conclusion, the EO was highly efficacious against L_1 but not against egg hatching. The action of this plant species against *H. contortus* is likely associated with dillapiole, the major EO component. Thus, the results obtained in the present study suggest that the EO derived from *P. aduncum* causes nematode toxicity via dillapiole-mediated cytochrome P450 inhibition in both isolates of *H. contortus*. We started from the premise that this EO, specifically dillapiole, could provide a natural source of anthelminthic agents. However, the differences observed in ICs indicate that the development of anti-parasitic drugs based on dillapiole, or other chemicals containing the methylenedioxyphenyl group, may be of little value. While the results indicate there may be a relationship between resistance status to commercially available chemicals and the plant-derived compound described in the present study, further research should expand the number of resistant and susceptible populations assessed in the bioassay.

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